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Up-regulation of CD14 in Liver Caused by Acute Ethanol Involves Oxidant-dependent AP-1 Pathway*

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Ethanol is known to cause both tolerance and sensitization to endotoxin (lipopolysaccharide). It is also known that ethanol modulates the expression and activity of several intracellular signaling molecules and transcription factors in monocytes and Kupffer cells, the resident hepatic macrophages. Expression of CD14, the endotoxin receptor, is up-regulated following chronic exposure to endotoxin and ethanol. Ethanol-induced oxidative stress is important in the regulation of transcription factor activation and cytokine production by Kupffer cells. Thus, it was hypothesized that acute ethanol increases CD14 expression through a mechanism dependent upon oxidant production. This hypothesis was tested by overexpression of superoxide dismutase via recombinant adenovirus. Mice were infected with adenovirus (3×10^9 plaque-forming units, intravenously) containing either Cu,Zn superoxide dismutase (Ad.SOD1) or β-galactosidase (Ad.*lacZ*), which caused significant expression of Cu,Zn-SOD in hepatocytes and Kupffer cells. Three days post-infection, mice were given saline or ethanol (5 g/kg, intragastrically). A significant increase in CD14 mRNA was observed 3 h after ethanol, and this increase was almost completely blocked in mice overexpressing Cu,Zn-SOD. Additionally, overexpression of SOD also blunted ethanol-induced activation of redox-sensitive transcription factors NFκB and AP-1 and production of cytokines. However, only inhibition of AP-1 with dominant-negative TAK1 but not NFκB by dominant-negative IκBα significantly blunted ethanol-induced increases in CD14, suggesting that AP-1 is important for CD14 transcriptional regulation. It is also shown here that NADPH oxidase is important in the increase in CD14 due to ethanol. Moreover, these data suggest that acute ethanol causes sensitization to endotoxin through mechanisms dependent upon oxidative stress.

Ethanol is known to cause sensitization to endotoxin (lipopolysaccharide (LPS)),[1] yet the mechanisms responsible for this observation are not clearly understood. It is known that ethanol modulates both the expression and activity of several intracellular signaling molecules and transcription factors in monocytes and Kupffer cells, the resident hepatic macrophages. For example, acute ethanol increases expression and activity of the interleukin-1 receptor-associated kinase in mouse Kupffer cells (IRAK) (1). Recently, the signaling pathways involved in CD14 receptor activation have been described (2, 3). Because CD14 is not a transmembrane-spanning receptor, it couples with the toll-like receptor 4 (Tlr-4) and initiates intracellular signaling cascades (2, 3). Specifically, the Tlr-4 protein activates the IRAK, which in turn activates the NFκB signaling cascade including NIK, IKK, IκB, and NFκB (4–6). Moreover, activation of Kupffer cell by LPS leads to an increase in oxidant (*i.e.* superoxide and nitric oxide) through NADPH oxidase and nitric-oxide synthase, respectively. Interestingly, oxidant production is required for full activation of LPS-induced NFκB (7), suggesting that oxidants are involved in the signaling mechanisms. It was recently demonstrated that overexpression of antioxidant superoxide dismutase in Kupffer cells blunts LPS-induced NFκB activation and TNFα production in Kupffer cells *in vitro* (8).

It has been reported that ethanol also alters the subunit composition of inflammatory transcription factor NFκB by inducing the formation of p50 homodimerization of NFκB (9). Independent studies have shown that both IRAK and transcription factors NFκB and AP-1 are redox-sensitive (10). Thus, it is conceivable that oxidant production affects key signaling molecules and transcription factors and that these alterations are involved in sensitization to LPS caused by acute ethanol.

Enomoto *et al.* (11) have described the phenomena whereby ethanol alters the response of Kupffer cells, the resident hepatic macrophages, to a subsequent insult by LPS *in vivo*. It was also shown that pretreatment of animals with nonabsorbable antibiotics completely prevents tolerance and sensitization caused by ethanol, suggesting the mechanism involves gut-derived LPS and activation of Kupffer cells (12). It was also reported that an increase in CD14 expression correlated with sensitization. Thus, it is hypothesized that Kupffer cells are activated initially by LPS to produce oxidants via NADPH oxidase, which activates redox-sensitive transcription factors leading to an increase in cytokine production and CD14 expression. This hypothesis was tested by overexpressing antioxidant Cu,Zn superoxide dismutase (SOD) in liver using recombinant adenovirus. It is also important to note that adenovirus transduces both hepatocytes and Kupffer cells *in vivo* (8). Here, it is reported that ethanol increases both NFκB and AP-1 activity as well as cytokine production, all of which are blunted by SOD overexpression. Ethanol-induced increases in CD14 mRNA are blunted significantly by SOD overexpression, consistent with the hypothesis that ethanol-induced sensitization is mediated in part by an increase in CD14, which is dependent upon oxidant production. Finally, it is demonstrated that inhibition

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[1] The abbreviations used are: LPS, lipopolysaccharide; IRAK, interleukin-1 receptor-associated kinase; TNFα, tumor necrosis factor α; SOD, superoxide dismutase; TAK, TGF-β receptor-associated kinase; pfu, plaque-forming units; EMSA, electrophoretic mobility shift assay; ALT, alanine transaminase; ANOVA, analysis of variance.

of AP-1 with dominant-negative TAK1, an up-stream kinase for c-*jun*, blunts ethanol-induced up-regulation of CD14. These data clearly support the hypothesis that ethanol exposure results in the up-regulation of CD14 through oxidant-dependent activation of AP-1.

MATERIALS AND METHODS

Animals and Treatment—Male C57Bl/6 mice (18–22 g, The Jackson Laboratory) were used for these experiments except for studies evaluating the role of NADPH oxidase and TNFα in CD14 mRNA regulation. For those studies, mice deficient in p47^{phox}, a required regulatory subunit of NADPH oxidase, and mice deficient in TNFα were used. The p47^{phox} knockout mice were originally obtained from Dr. Steve Holland (13). The TNFα knockout mice were purchased as breeding pairs (The Jackson Laboratory), and colonies have been maintained within IACUC (Institutional Animal Care and Use Committee)-approved facilities and guidelines. C57Bl/6 were infected with adenovirus (1×10^9 plaque-forming units/animal, intravenously) 3 days prior to receiving either saline or ethanol (5 g/kg, intragastrically).

Adenoviral Synthesis and Preparation—Recombinant adenovirus containing the transgene for either Cu,Zn-SOD (Ad.SOD1) or β-galactosidase (Ad.*lacZ*) was prepared as described elsewhere (14, 15). Briefly, the plasmid shuttle vector pAd5.CMV.*lacZ* was constructed by standard cloning protocols as described by Sambrook *et al*. (16). The adenoviral shuttle plasmid was transfected into the permissive HEK-293 host cell line to generate recombinant Ad.*lacZ* adenovirus. Recombinant adenovirus containing the transgene for human Cu,Zn superoxide dismutase (Ad.SOD1) was the kind gift of Dr. John Engelhardt (University of Iowa). Preparation of adenovirus containing the transgene for hemagglutinin-tagged IκBα super-repressor (Ad.IκB) has been described previously (17). The IκBα super-repressor is a dominant-negative protein that contains Ser^{32} → Ala and Ser^{36} → Ala mutations, which inhibit phosphorylation and prevent NFκB dissociation and translocation into the nucleus. Recombinant adenoviruses containing dominant-negative TAK, an inhibitor of LPS-induced AP-1 activation (18), was provided by Dr. Richard Rippe (University of North Carolina). The virus isolates were plaque-purified and propagated in HEK-293 cells, isolated, concentrated, and titered by plaque assay. Purified recombinant adenovirus (1×10^9 plaque-forming units) was suspended in normal saline and injected into animals via the tail vein.

Liver Cell Isolation—Kupffer cells and hepatocytes were isolated from naive Sprague-Dawley rats (250–300 g) or rats infected with Ad.*lacZ* (1×10^9 pfu) 3 days earlier. Briefly, livers were isolated following pentobarbital anesthesia (60 mg/kg, intraperitoneally) and perfused via the portal vein for 10 min with Krebs-Ringer-HEPES buffer containing 115 mM NaCl, 5 mM KCl, 1 mM KH_2PO_4, 25 mM HEPES, 1 mM $CaCl_2$, and 0.016% collagenase (pH 7.4) followed by 10 min of perfusion with calcium-free buffer containing 0.5 mM EGTA. Liver cells were dispersed by gentle shaking in phosphate-buffered saline (pH 7.4, 4 °C), and the nonparenchymal cell fraction was separated from parenchymal cells by centrifugation through Percoll gradients based on a method developed by Smedsrod and Pertoft (19).

Western Blot Analysis—Either whole liver or isolated hepatocytes and Kupffer cells were homogenized, and samples (30 μg) were resolved by electrophoresis using 12% SDS-PAGE. Proteins were blotted with either anti-β-galactosidase antibody (Roche Molecular Biochemicals) or anti- Cu,Zn-SOD antibody (Oxis, Portland, OR), followed by horseradish peroxidase-conjugated secondary antibody. The anti-SOD antibody is cross-reactive with both endogenous rat Cu,Zn-SOD and the human recombinant SOD. Protein was visualized by radiography using ECL Western detection reagent (Amersham Biosciences).

Immunohistochemical Staining for CD14—Formalin-fixed, paraffin-embedded sections (6 μm) were mounted on glass slides. Sections were deparaffinized, rehydrated, and then stained with mouse anti-CD14 primary antibody (Santa Cruz Biotechnology) for 30 min. The immunostaining was visualized using the DAKO immunostaining kit. Slides were counterstained with hematoxylin. Primary antibody dilutions were 1:500 in phosphate-buffered saline containing 1% Tween 20.

Electomobility Shift Assay—For studies in whole liver, nuclear extracts were isolated as described by Dignam *et al.* (20) with minor modifications (21). Binding conditions for NFκB and AP-1 were characterized and EMSA was performed as described elsewhere (22, 23). Briefly, nuclear extracts (20 μg) from liver tissue were preincubated with 1 μg of poly(dI-dC), 20 μg bovine serum albumin (Amersham Biosciences) and 2 μl of a ^{32}P-labeled DNA probe (10,000 cpm/μl) containing 1 ng of double-stranded oligonucleotide in a total volume of 20 μl. Mixtures were incubated FOR 20 min on ice and resolved on 5% polyacrylamide (29:1 cross-linking) and 0.4× TBE (Tris borate-EDTA) gels. After electrophoresis, gels were dried and exposed to X-Omat LS Kodak film. The intensity of NFκB and NFκB binding was quantitated by scanning autoradiograms with GelScan XL (Amersham Biosciences) and is expressed as arbitrary densitometric units.

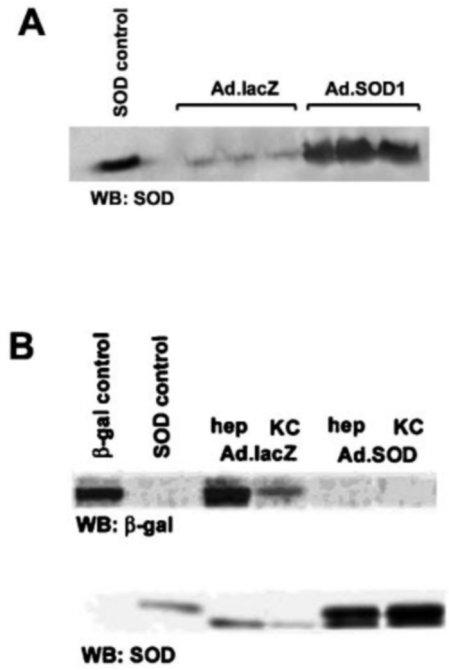

FIG. 1. **Expression of SOD in hepatocytes and Kupffer cells.** Mice were infected with either Ad.*lacZ* or Ad.SOD1 (1×10^9 pfu). Three days after the injection, livers were harvested. *A*, whole liver extracts were separated by 12% SDS-PAGE, and SOD expression was determined by Western blot (*WB*) analysis using anti-Cu,Zn-SOD antibody. Each lane represents extract from individual mice. *B*, hepatocytes and Kupffer cells were isolated from mice infected with either Ad.*lacZ* or Ad.SOD1 as described under "Materials and Methods." Extracts were separated by 16% SDS-PAGE followed by immunoblotting with a monoclonal antibody against β-galactosidase or Cu,Zn-SOD. Lysates from human embryonic kidney (HEK-293) cells infected with Ad.*lacZ* and Ad-SOD1 were used as positive controls.

RNase Protection Assay—Total RNA was isolated from liver tissue using RNA-STAT 60 (Tel-Test, Friendswood, TX). RNase protection assays for cytokine expression were performed using the RiboQuant multiprobe assay system (Pharmingen). Briefly, [^{32}P] RNA probes were transcribed with T7 polymerase using the multiprobe template set mCK-3 (Pharmingen). For CD14, a cDNA fragment amplified by reverse transcriptase PCR from mouse macrophage cDNA library was subcloned into pCR-Topo (Stratagene, Cedar Creek, TX) by standard cloning procedures. Prior to translation of the RNA probe, the pCR plasmid containing the CD14 insert was linearized by *Hin*dIII restriction digest. The RNA probe was generated with T7 polymerase using the transcription assay described above. RNA (20 μg) was hybridized with 4×10^5 cpm of probe overnight at 56 °C. Samples were then digested with RNase followed by proteinase K treatment, phenol:chloroform extraction, and ethanol precipitation. Samples were resolved on a 5% acrylamide-bisacrylamide (19:1) urea gel. After drying, the gel was visualized by autoradiography.

RESULTS

The Overexpression of SOD in Nonparenchymal and Parenchymal Cells—To evaluate gene transfer of SOD in these studies, whole liver extracts were evaluated by Western blot using antibodies against the human isoform of Cu,Zn-SOD (Fig. 1*A*). In the livers of animals 3 days after Ad.*lacZ* infection, low levels of human Cu,Zn-SOD were detected, which was most likely caused by cross-reactivity with the mouse isoform. In Ad.SOD1-infected mice, however, significant Cu,Zn-SOD expression was observed.

It was demonstrated that recombinant adenovirus could transduce both hepatocytes and Kupffer cells *in vivo* (8). Thus,

experiments were performed to determine whether SOD was overexpressed in each cell type following Ad.SOD1 infection in mice. Mice were infected with either Ad.*lacZ* or Ad.SOD1 as described above, and Kupffer cells and hepatocytes were isolated 3 days after infection as described under "Materials and Methods." Lysates from each cell type were separated by SDS-PAGE and immunoblotted for either Cu,Zn-SOD or β-galactosidase (Fig. 1*B*). β-Galactosidase was detected in hepatocytes from Ad.*lacZ*-infected animals but not in hepatocytes from Ad.SOD1-infected mice. Similarly, β-galactosidase expression was observed in Kupffer cells from Ad.*lacZ*-infected animals but not in those from Ad.SOD1-infected mice. As expected, Cu,Zn-SOD expression in hepatocytes was significantly increased in mice infected with Ad.SOD1 compared with mice infected with Ad.*lacZ*. Kupffer cells from Ad.*lacZ*-infected mice expressed very little Cu,Zn-SOD. However, Cu,Zn-SOD expression was significantly increased in Kupffer cells from mice infected with Ad.SOD1, confirming that Ad.SOD1 transduces both hepatocytes and Kupffer cell *in vivo*, resulting in overexpression of SOD in each cell type.

Ethanol-induced Sensitization to LPS in Mouse Liver Is Blunted by Overexpression of SOD—It is known that ethanol enhances the response of mice to LPS administration. The hypothesis that acute oxidative stress plays a role in the mechanisms of sensitization was tested here. First, C57Bl/6 mice were given either saline or ethanol (5 g/kg, intragastrically) followed by LPS (2.5 mg/kg, intravenously) 24 h later. Serum alanine transaminase (ALT) levels were measured at 0, 1, 2, 4, and 8 h after LPS administration (Fig. 2*A*). As expected, serum ALT levels were elevated in saline-pretreated animals at 8 h after LPS at 110 ± 12 units/ml. However, in ethanol-pretreated mice, the increase in serum ALT levels 8 h after LPS was significantly potentiated by nearly 80% (186 ± 12 units/ml, $p < 0.05$, Repeated measures ANOVA) mice. These data support the hypothesis that ethanol induces sensitization to LPS, which is consistent with numerous reports.

Next, animals were infected with recombinant adenovirus (1×10^9 pfu/animal, intravenously) containing either Cu,Zn-superoxide dismutase (Ad.SOD1) or β-galactosidase (Ad.*lacZ*). Three days after infection, when transgene expression is optimal, animals were given either saline or ethanol (5 g/kg, intragastrically), followed 24 h later by LPS (2.5 g/kg, intravenously) or vehicle alone. The increase in serum ALT levels caused by LPS alone was slightly blunted in Ad.SOD1-infected mice but was not significantly different after 8 h compared with levels in Ad.*lacZ*-infected animals (Fig. 2*B*). As expected, ethanol pretreatment of Ad.*lacZ*-infected animals given LPS resulted in a significant increase in serum ALT release over Ad.*lacZ*-infected animals pretreated with saline. Importantly, under these conditions, ethanol alone in both Ad.*lacZ*- and Ad.SOD1-infected animals did not cause a significant increase in ALT release. However, in animals infected with Ad.SOD1, ethanol pretreatment had no effect on LPS-induced increase in serum ALT levels. These data strongly support the hypothesis that oxidative stress participates in the mechanisms of sensitization to LPS induced by ethanol in mouse liver.

Overexpression of SOD Blunts Acute Ethanol-induced Redox-sensitive Transcription Factor Activation—To understand how oxidants participate in the mechanisms of ethanol-induced sensitization to LPS, the hypothesis that oxidants are involved in activation of the redox-sensitive transcription factors NFκB and AP-1 following acute ethanol was tested. First, control mice were given ethanol (5 g/kg, intragastrically), liver nuclear extracts were isolated 0, 1, 3, and 6 h later, and the DNA binding activity of both NFκB and AP-1 was evaluated by EMSA (Fig. 3). Peak NFκB DNA binding activity was observed ~3 h after ethanol

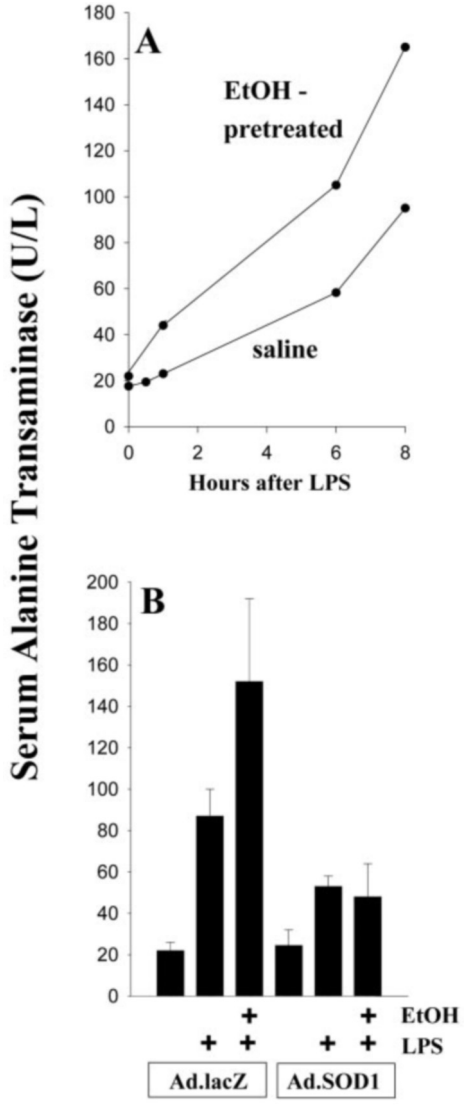

FIG. 2. **Overexpression of SOD blunts acute ethanol-induced sensitization to LPS.** *A*, mice (22–25 g, C57Bl/6) were given LPS (2.5 mg/kg) 21 h after receiving ethanol (5 g/kg, intragastrically) or saline. Serum was collected at intervals of up to 8 h after LPS, and serum transaminase levels were measured as described under "Materials and Methods." *B*, mice (22–25 g, C57Bl/6) were infected with recombinant adenovirus (1×10^9 pfu/animal) containing either Cu,Zn-SOD or β-galactosidase. Three days after infection, mice were given ethanol (5 g/kg, intragastrically) or saline followed by LPS (2.5 mg/kg) after 21 h. Serum was collected at intervals of up to 8 h after LPS, and serum transaminase levels were measured as described under "Materials and Methods."

exposure (Fig. 3*A*). Using nuclear extracts isolated 3 h after ethanol exposure, competition and supershift assays were performed to validate the specificity of NFκB DNA binding. The addition of unlabeled probe completely blocked DNA binding in extracts from ethanol-treated mice. Moreover, the addition of antibodies against mouse p50 and p65 subunits of NFκB caused retardation in NFκB gel shift mobility, confirming the formation of active NFκB induced by ethanol exposure. Similar studies were performed to evaluate the activation of AP-1 following acute ethanol. The DNA binding activity of AP-1 also peaked at ~3 h after ethanol (Fig. 3*B*). The specificity of AP-1 DNA binding was also determined by competition and supershift assays using antibodies against both c-Fos and c-Jun.

To test the hypothesis that overexpression of Cu,Zn-SOD would blunt acute ethanol-induced NFκB and AP-1 activation, mice were infected with recombinant Ad.*lacZ* (1×10^9 pfu/

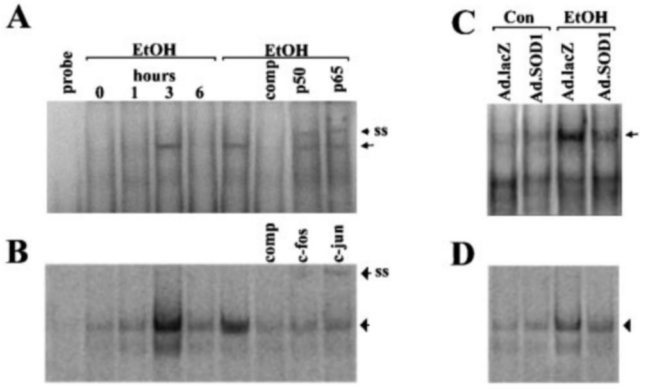

FIG. 3. **Acute ethanol-induced redox-sensitive transcription factor activation is blunted by SOD.** *A*, mice were given ethanol (5 g/kg, intragastrically) and sacrificed after 0, 1, 3, and 6 h. Nuclear extracts (20 μg) prepared from whole liver were used to evaluate NFκB DNA binding activity by EMSA as described under "Materials and Methods." Using nuclear extract isolated from mice 3 h after ethanol exposure, NFκB competition assay using 100-fold excess unlabeled oligo (*comp*), and supershift assays using 4 μl of p50 and p65 antibodies (Santa Cruz Biotechnology) were performed. *B*, AP-1 DNA binding activity was determined by EMSA using nuclear extract as described above. Competition was performed using excess unlabeled AP-1 oligo, and supershifts were done using 4 μl of antibody against either c-*fos* or c-*jun* (Santa Cruz Biotechnology). *Arrows* refer to specific DNA binding; *ss*, supershifted DNA-binding complex. *C*, mice were infected with recombinant adenovirus (1×10^9 pfu/animal) containing either β-galactosidase (Ad.*lacZ*) or Cu,Zn-SOD (Ad.SOD1). Three days after infection, mice received ethanol (5 g/kg, intragastrically) or saline (*Con*) and sacrificed 3 h later. NFκB was measured by EMSA as described under "Materials and Methods." *D*, AP-1 activity was measured 3 h after ethanol administration in mice infected with either Ad.*lacZ* or Ad.SOD1. Gels shown are representative of three individual experiments.

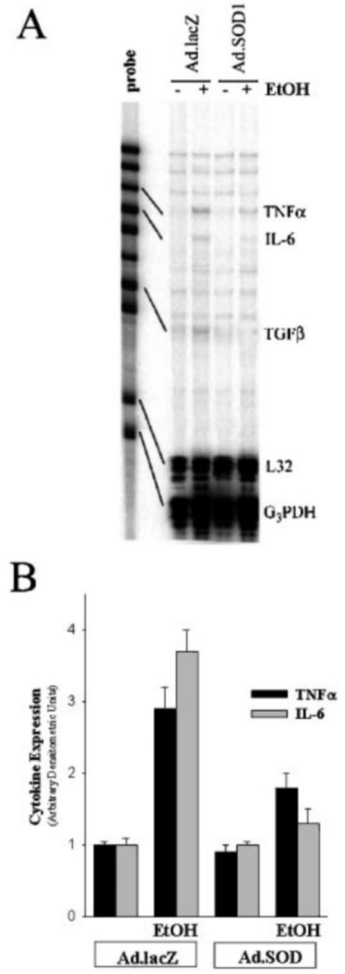

FIG. 4. **Increase in cytokine mRNAs after acute ethanol administration.** *A*, whole liver RNA was isolated from animals infected with Ad.*lacZ* or Ad.SOD1 (1×10^9 pfu/animal) 3 h after ethanol (5 g/kg, intragastrically) or saline administration. Cytokine expression was measured by RNase protection assay as described under "Materials and Methods." *B*, image analysis was performed on an RNase protection assay from the experiments described above using an ImageQuant PhosphorImager. Data are expressed as means ± S.E. and are representative of three experiments in each group. (*, $p < 0.05$, two-way ANOVA, Tukey's post hoc analysis).

animal) or Ad.SOD1 as described above. Three days after infection, mice were given 5 g/kg ethanol, and nuclear extracts were isolated from liver 3 h later. Ethanol caused a significant increase in NFκB activity in Ad.*lacZ*-infected mice. Importantly, ethanol-induced NFκB activation was markedly blunted in mice infected with Ad.SOD1 (Fig. 3*C*). In animals infected with Ad.*lacZ*, ethanol induced a significant increase in AP-1 activity, which was not observed in animals infected with Ad.SOD1 (Fig. 3*D*). These data strongly support the hypothesis that oxidant generation caused by exposure to acute ethanol is important for activation of redox-sensitive transcription factor AP-1. Moreover, these data suggest that activation of NFκB and AP-1 may be important in the sensitization to LPS caused by acute ethanol exposure.

Cytokine Production Caused by Acute Ethanol Exposure Is Blunted by SOD—NFκB and AP-1 are known transcription factors that regulate the expression of several inflammatory cytokines. Thus, mRNA levels of cytokines were measured in livers of Ad.*lacZ*- and Ad.SOD1-infected mice following administration of either saline or ethanol (Fig. 4). Consistent with transcription factor activation, mRNA levels for TNFα were significantly elevated in Ad.*lacZ*-infected animals following ethanol injection. In Ad.SOD1-infected mice, the ethanol-induced increase in TNFα mRNA was blunted by >70%. Similarly, ethanol caused an increase in interleukin-6 mRNA levels, which was nearly completely blocked in Ad.SOD1-infected mice. These changes in mRNA, quantified by phosphorimaging, clearly demonstrate that oxidants are involved in the acute ethanol-induced increase in cytokine production.

To verify these findings, serum cytokine levels were measured in mice following ethanol exposure. In animals infected with Ad.*lacZ*, a peak increase in serum TNFα levels was observed 6 h after ethanol administration. Ethanol caused a significant increase in TNFα from basal levels of 28.1 ± 4.3 pg/ml to 46.7 ± 7.4 pg/ml ($p < 0.05$, two-way ANOVA, Tukey's post hoc analysis). This increase in serum TNFα was blunted to 29.4 ± 6.3 pg/ml in mice infected with Ad.SOD1. These data correlate with the ethanol-induced changes in TNFα mRNA levels in mice infected with Ad.*lacZ* and Ad.SOD. Surprisingly, interleukin-1 or interleukin-6 was not detected in serum following ethanol exposure in either Ad.*lacZ*- or Ad.SOD1-infected mice. Perhaps this is related to changes in localized expression of these cytokines, which may not alter serum levels.

Acute Ethanol Increases CD14 Expression—Recently it was demonstrated that chronic ethanol-fed animals exhibited high levels of CD14 expression on Kupffer cells (12, 25). The hypothesis is that ethanol exposure causes an up-regulation of CD14, which "primes" Kupffer cells to LPS causing an elevated inflammatory response. Moreover, promoter elements for the CD14 gene have been shown to contain several AP-1 consensus binding sites (26, 27). Thus, the hypothesis that acute ethanol increased the expression of CD14 in whole liver was tested. Animals were given ethanol (5 g/kg, intragastrically) and sacrificed after 0, 1, 3, 12, and 21 h. CD14 mRNA, measured by RNase protection assay, was elevated significantly at 3 h after

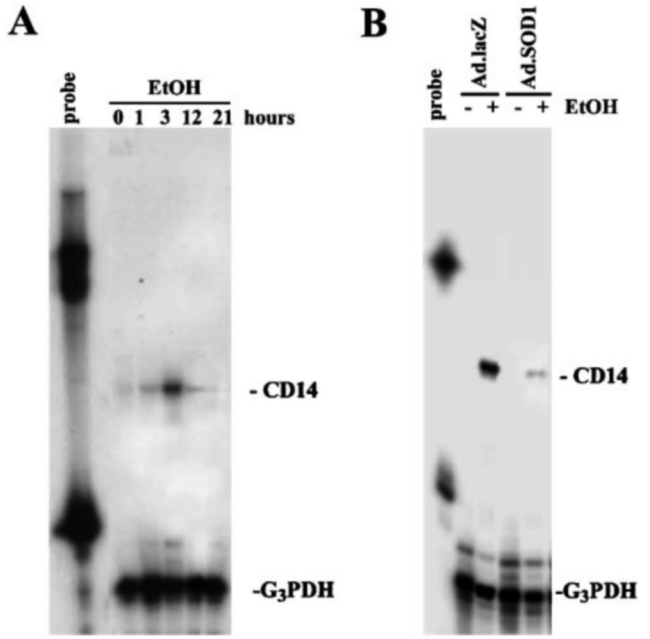

FIG. 5. **Ethanol-induced CD14 expression is blunted by SOD.** *A*, mice were given ethanol (5 g/kg, intragastrically) and sacrificed at 0, 1, 3, 12, and 21 h after ethanol exposure. CD14 mRNA was then measured by RNase protection assay. *B*, mice, treated as described in the legend for Fig. 4, were sacrificed at 3 h after ethanol or saline exposure. CD14 mRNA levels were measured by RNase protection assay as described under "Materials and Methods." These data are representative of three individual experiments.

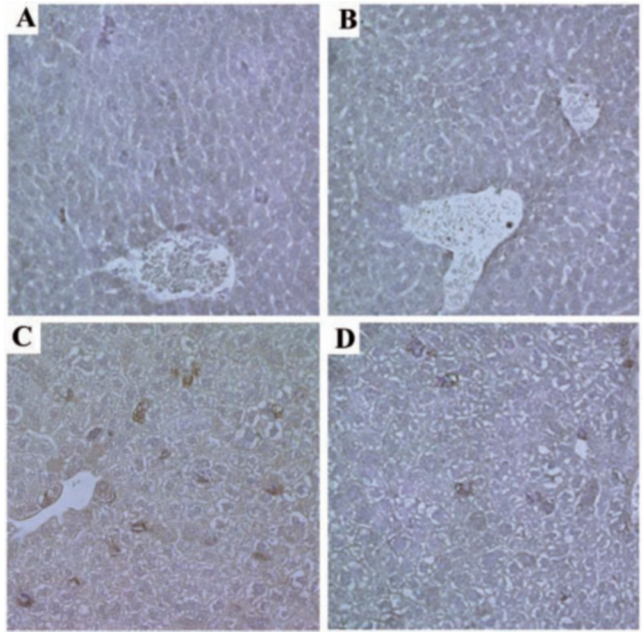

FIG. 6. **Localization of CD14 expression.** Mice (22–25 g, C57Bl/6) were infected with recombinant adenovirus (1×10^9 pfu/animal) containing either β-galactosidase (*A* and *C*) or Cu,Zn-SOD (*B* and *D*). Three days after infection, mice were given saline (*A* and *B*) or ethanol (*C* and *D*) (5 g/kg, intragastrically). 24 h later, mice were sacrificed and livers were harvested. Liver sections were stained immunohistochemically for CD14 using antibodies against mouse CD14 (Santa Cruz Biotechnology). Data are representative of four individual experiments.

ethanol administration (Fig. 5*A*), supporting the hypothesis that ethanol increases CD14 expression *in vivo*.

To test the hypothesis that oxidants were involved in ethanol-induced up-regulation of CD14, mice were infected with Ad.SOD1 (1×10^9 pfu/animal) or Ad.*lacZ* 3 days prior to ethanol (5 g/kg, intragastrically) or saline treatment, and CD14 mRNA was measured 3 h later. Compared with saline, ethanol caused a significant increase in CD14 mRNA in Ad.*lacZ*-infected animals. However, the increase in CD14 induced by ethanol was dramatically blunted in animals infected with Ad.SOD1 (Fig. 5*B*). These data strongly support the hypothesis that oxidants are involved in acute ethanol-induced increases in CD14 levels. The strong correlation between the inhibition of redox-sensitive transcription factors and CD14 up-regulation suggests that CD14 expression may be regulated by NFκB and/or AP-1.

Because it has been reported that CD14 can be expressed on hepatocytes in addition to Kupffer cells, it was important to determine the location of CD14 expression following acute ethanol exposure. To test the hypothesis that ethanol caused an up-regulation of CD14 primarily on Kupffer cells, liver sections from mice infected with either Ad.*lacZ* or Ad.SOD1 and given either saline or ethanol were stained immunohistochemically for CD14 (Fig. 6). Compared with results in saline-treated mice, a significant increase in CD14 detection was observed 24 h after ethanol administration. CD14 expression was increased predominantly in sinusoidal cells (*i.e.* Kupffer cells). Ethanol-induced CD14 expression was not significantly increased over basal levels of expression in mice infected with Ad.SOD1. This is an important finding confirming that an increase in CD14 mRNA translates into increased protein expression and that ethanol-induced expression is most likely limited to Kupffer cells within the liver.

Inhibition of AP-1 Blunts Ethanol-induced Up-regulation of CD14—Because AP-1 regulatory sites are present in the promoter region of CD14 (27), it was hypothesized that AP-1 regulates CD14 expression following acute ethanol administration. To test this hypothesis, animals were infected with an adenovirus (1×10^9 pfu/animal, intravenously) containing the transgene for either β-galactosidase, dominant-negative IκBα, an inhibitor of NFκB, or dominant-negative TAK1 (Ad.ΔTAK), an up-stream activation kinase for AP-1 (18). Three days after infection, animals were given 5 g/kg ethanol as above and sacrificed 3 h later, and CD14 mRNA levels were measured by RNase protection assay (Fig. 7). As expected, in Ad.*lacZ*-infected animals, ethanol caused a significant increase in CD14 mRNA compared with animals given saline. Overexpression of dominant-negative IκBα, a specific inhibitor of NFκB, had no effect on ethanol-induced up-regulation of CD14, despite a clear inhibition of NFκB activation *in vivo* (data not shown). However, the increase in CD14 mRNA levels following ethanol was dramatically blunted in animals infected with Ad.ΔTAK, strongly supporting the hypothesis that AP-1 activation is involved in the up-regulation of CD14 under these conditions. Also, overexpression of dominant-negative TAK significantly blunted ethanol-induced activation of AP-1 DNA binding compared with controls (data not shown).

NADPH Oxidase Is Necessary for Acute Ethanol-induced Increases in CD14—Because oxidants are involved in acute ethanol-induced CD14 expression *in vivo*, and CD14 is primarily expressed on Kupffer cells, it is hypothesized that NADPH oxidase, the primary source of oxidants in Kupffer cells, is important in the mechanism. Thus, ethanol (5 g/kg, intragastrically) or saline was given to either wild-type or NADPH oxidase-deficient mice ($p47^{phox-/-}$), and CD14 expression was measured 3 h later. As expected, in wild-type mice, administration of ethanol, as compared with saline, caused a significant increase in CD14 mRNA (Fig. 8*A*). However, ethanol-induced CD14 expression was blunted by >75% in NADPH oxidase-deficient mice. These data strongly suggest that NADPH oxidase is central to oxidant production following acute ethanol and that it is involved in the up-regulation of CD14, most likely through NFκB and AP-1 transcription factors.

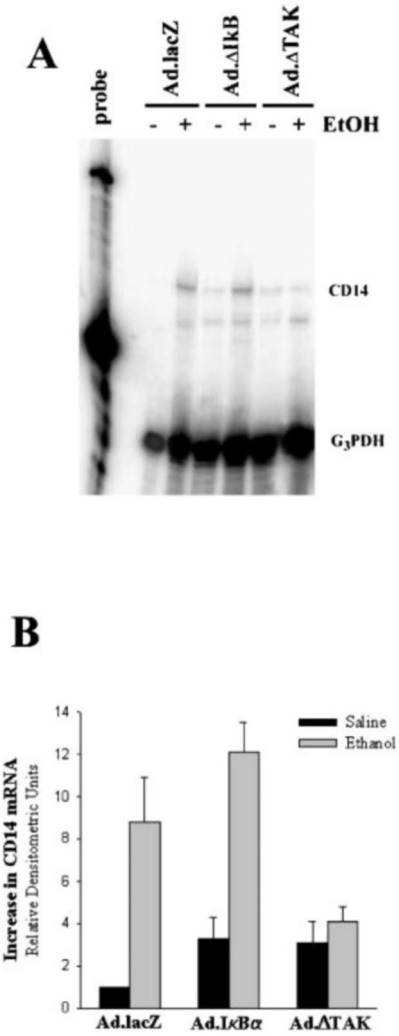

FIG. 7. **Inhibition of AP-1 blunts ethanol-induced CD14 up-regulation.** *A*, mice were infected with recombinant adenovirus (1×10^9 pfu/animal) containing β-galactosidase (*Ad.lacZ*), dominant-negative IκBα super-repressor (*Ad.IκBα*), or dominant-negative TAK (*Ad.ΔTAK*). Three days after infection, mice received ethanol (5 g/kg, intragastrically) or saline and were sacrificed 3 h later. CD14 mRNA was evaluated by RNase protection assay as described under "Materials and Methods." *B*, image analysis was performed using an ImageQuant PhosphorImager. Data are expressed as means ± S.E. and are representative of three experiments in each group. (*, $p < 0.05$, two-way ANOVA, Tukey's post hoc analysis).

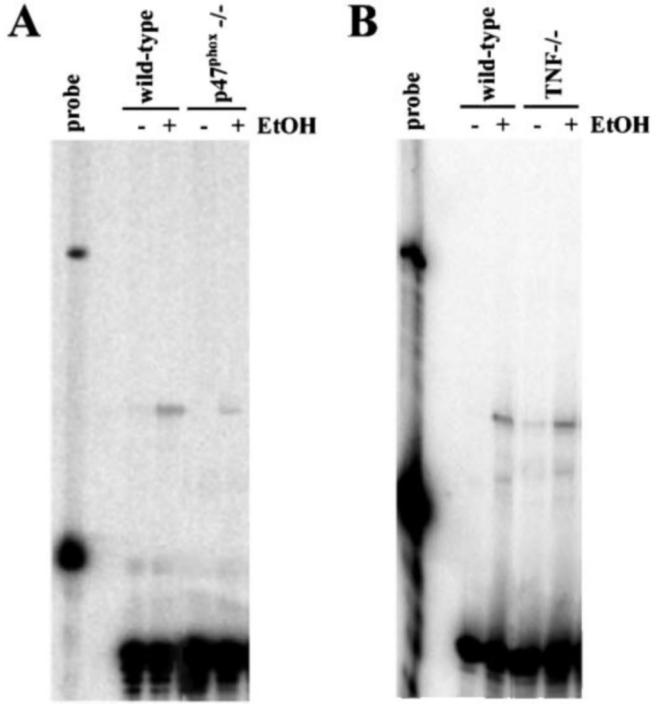

FIG. 8. **NADPH oxidase, but not TNFα, is required for ethanol-induced increase in CD14.** Mice deficient in $p47^{phox}$, a critical regulatory subunit of NADPH oxidase ($p47^{phox}-/-$), TNFα (*TNF*−/−), or wild-type control mice were given ethanol (5 g/kg, intragastrically) or saline, and CD14 mRNA was evaluated 3 h later by RNase protection assay as described under "Materials and Methods." Data are expressed as means ± S.E. and are representative of three experiments in each group. (*, $p < 0.05$, two-way ANOVA, Tukey's post hoc analysis).

TNFα Signaling Is Not Required for Ethanol-induced Increases in CD14—Because acute ethanol causes TNFα production and also oxidative stress, we hypothesized that TNFα mediates AP-1 activation and up-regulation of CD14. To test this hypothesis, mice deficient in TNFα (TNF−/−) and wild-type control mice were given either saline or ethanol (5 g/kg, intragastrically). Three hours later, the mice were sacrificed, and the level of CD14 mRNA was measured by RNase protection assay (Fig. 8*B*). In the wild-type controls, ethanol induced a significant increase in CD14 expression compared with wild-type mice receiving only saline. Similarly, ethanol caused a significant increase in CD14 mRNA in TNFα−/− mice. Importantly, the increase in CD14 mRNA due to ethanol was not different between wild-type and TNFα−/− mice, suggesting that TNFα signaling is not required for the increase in CD14 expression under these conditions.

DISCUSSION

Role of CD14 in Ethanol-induced Liver Disease—A recently described polymorphism in the human CD14 promoter region, resulting in higher levels of CD14, is associated with an increase in severity of alcoholic liver disease (28). These human studies, along with several animal studies, strongly support the involvement of CD14 in alcoholic liver disease and suggest that CD14 is a risk factor for ethanol-induced pathology (29). CD14, which is expressed largely on Kupffer cells in the liver, is activated by circulating endotoxin that enters the liver via the portal vein. CD14 is a membrane-associated receptor but has no transmembrane-spanning region. Thus, it associates with the toll-like receptor-4, which initiates intracellular signaling. Although clearly defined signaling pathways have not been identified, activation of CD14 results in the production of superoxide free radicals and toxic cytokines, including TNFα, as well as a number of other inflammatory responses. Mice deficient in CD14 are almost completely resistant to LPS-induced liver injury at physiological levels (*i.e.* less than 10–50 pg/ml). Moreover, CD14-deficient mice do not exhibit any pathological changes due to chronic ethanol exposure compared with wild-type mice. Together, these findings demonstrate the importance of CD14 and endotoxin in ethanol-induced liver injury. Activation of CD14 on Kupffer cells leads to the production of superoxide and TNFα, which are involved in the pathogenesis of alcoholic liver disease.

Up-regulation of CD14 in Sensitization—We and others have shown that CD14 levels are elevated in liver following chronic exposure to ethanol (1, 24, 25). This increase correlates with ethanol-induced liver injury. Thus, steady increases in CD14 expression because of ethanol exposure, resulting in increased cytokine production, could exacerbate ethanol-induced injury. These studies were done to understand the mechanisms involved in CD14 up-regulation due to ethanol. It is shown here that acute ethanol administration leads to a rapid increase in CD14 expression *in vivo*. These data are consistent with the

observation that acute ethanol causes hypersensitivity to LPS, a mechanism known as "priming" or "sensitization." It is hypothesized that ethanol activates signaling mechanisms that result in the up-regulation of CD14 on Kupffer cells, which sensitizes animals to subsequent challenge with LPS. Because it is known that ethanol activates redox-sensitive transcription factors NFκB and AP-1 and induces a rapid increase in CD14 expression, it is hypothesized that oxidants activate either NFκB or AP-1 and increase the synthesis of CD14. Moreover, it was hypothesized that overexpression of SOD would blunt the increase in CD14 due to acute ethanol. In these experiments it was clearly demonstrated that SOD blunted the priming effect of ethanol *in vivo* (Fig. 2*B*). However, it is difficult to understand whether SOD directly blunts the effects of ethanol on priming or whether SOD blunts LPS signaling itself. Therefore, studies were done to evaluate the role of oxidants in the priming effect of ethanol alone. Consistent with the hypothesis that ethanol primes the liver, acute ethanol activates NFκB and AP-1 (Fig. 3). Acute ethanol also causes a transient increase in cytokine production similar to the increase in CD14 expression (Figs. 4 and 5). Thus, it was also hypothesized that activation of transcription factors NFκB and AP-1 played a role in the up-regulation of CD14.

Activation of AP-1 and Up-regulation of CD14—To determine whether AP-1 was directly responsible for the up-regulation of CD14, studies were done to inhibit AP-1 using dominant-negative TAK expression via adenovirus. Inhibition of AP-1, either indirectly by overexpression of SOD or directly by dominant-negative TAK, blunted the increase in CD14 expression due to acute ethanol in addition to blunting AP-1 activation. TAK has been demonstrated to be an "up-stream" kinase of AP-1, leading to its activation (18). These data are consistent with the hypothesis that AP-1 is a critical transcription factor involved in the up-regulation of CD14. This finding is consistent with other reports demonstrating that the CD14 promoter contains several AP-1-responsive elements (26). Overexpression of superoxide dismutase blunted ethanol-induced activation of NFκB and AP-1 and up-regulation of CD14, suggesting that these redox-sensitive transcription factors are involved in the regulation of CD14. However, it is likely that AP-1 and not NFκB is actually involved because NFκB regulatory elements within the CD14 promoter have not been identified. Moreover, inhibition of NFκB using the dominant-negative IκBα superrepressor, although blocking ethanol-induced NFκB activation, had no effect on the up-regulation of CD14 following acute ethanol (Fig. 7). On the other hand, inhibition of AP-1 activation using dominant-negative TAK completely and significantly blunted the increase in CD14 expression (Fig. 7).

It is hypothesized that TNFα release by Kupffer cells in response to acute ethanol activates the AP-1 pathway, leading to an increase in CD14 expression. However, we demonstrate here that the up-regulation of CD14 was not affected in mice deficient in TNFα (Fig. 8). These data suggest that TNFα signaling is not necessary for ethanol to up-regulate CD14. This is an important point because it was shown that TNFα is required for long-term ethanol induced liver injury. Interestingly, deletion of NADPH oxidase in p47^{phox} mutant mice did inhibit CD14 up-regulation due to ethanol, supporting the hypothesis that NAPDH oxidase is an early source of oxidants due to ethanol. This experiment was performed to identify the potential source of oxidant production that contributed to AP-1 activation and CD14 expression. The p47^{phox} subunit is most likely a critical regulatory factor of NADPH oxidase, based on a number studies. Therefore, these data suggest that NADPH oxidase is important for oxidant production following acute ethanol. However, with the expanding information about NADPH oxidase genes, it is becoming clear that p47^{phox} expression is not limited to macrophages, unlike the catalytic subunit gp91^{phox} of NADPH oxidase, which is predominantly expressed on macrophages. The role of gp91^{phox} in ethanol-induced oxidative stress in liver is an important unresolved question.

In conclusion, ethanol induces oxidant production through NADPH oxidase in Kupffer cells leading to the activation of redox-sensitive transcription factors, which simultaneously upregulates cytokine expression and CD14 expression. It has recently been shown that CD14 is necessary for ethanol-induced liver injury in mice and that increased levels of CD14 correlate with severity of alcoholic liver disease in humans. Thus, it is very likely that acute doses of ethanol cause an increase in CD14 and that progressive increases in CD14 underlie the mechanisms of pathology. As ethanol exposure increases in CD14 expression, a hyper-responsiveness may lead to a significant and sustained increase in oxidant and cytokine production, which disposes the liver to greater injury. Although CD14 is required for ethanol-induced liver injury, it is not clear whether basal levels of CD14 are sufficient to mediate the pathological effects of ethanol and endotoxin or whether the increase in CD14 expression is required. This is an intriguing question because it represents a potential target for therapy, to inhibit the up-regulation of CD14 *in vivo*.

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